

## APPENDIX B

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# Sensitive procedure for the determination of reboxetine enantiomers in human plasma by reversed-phase high-performance liquid chromatography with fluorimetric detection after chiral derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate

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## ABSTRACT

A sensitive and selective high-performance liquid chromatographic method for the determination of reboxetine enantiomers in human plasma was developed. Although two chiral centres are present in reboxetine, its stereospecific synthesis leads to two rather than four possible enantiomers. After extraction from plasma and reaction with (+)-1-(9-fluorenyl)ethyl chloroformate, reboxetine enantiomers were separated as diastereoisomeric derivatives by reversed-phase high-performance liquid chromatography (HPLC) and determined by fluorimetric detection. The HPLC analysis time was about 90 min. The linearity, precision, accuracy and limit of quantification of the method were evaluated. No interference from blank plasma sample was observed. The suitability of the method for *in vivo* samples was assessed by the analysis of plasma samples obtained from a healthy male volunteer who had received a single oral dose of 4 mg of reboxetine in tablet form.

## INTRODUCTION

Reboxetine, (*RS*)-2-[(*RS*)- $\alpha$ -(2-ethoxyphenoxy)benzyl]morpholine methanesulphonate [laboratory code FCE 20124, (*I*)] is a compound developed by Farmitalia Carlo Erba (Milan, Italy) which has shown high potency in classical pharmacological models predictive of antidepressant efficacy in humans: reserpine antagonism and norepinephrine reuptake inhibition [1]. In addition, *I* has been found to prevent clonidine-induced hypothermia in rodents after a single oral dose [1]. When the effects of *I* on the resting pupil diameter and the pupillary responses to light stimuli were studied in healthy volunteers, the results obtained probably reflected blockage of norepinephrine uptake and of muscarinic

receptors by *I* [2]. The compound is extensively metabolized in rodents, where no unchanged drug is recovered in the urine, whereas some unchanged *I* is detected in the urine of dog and monkey, although less than in humans [3]. *I* shows important structural similarities with viloxazine, nisooxetine and fluoxetine. With viloxazine and nisooxetine it also shares the property of being a selective norepinephrine uptake inhibitor, whereas it shows only structural similarities with fluoxetine, which is a selective serotonin uptake inhibitor. Two chiral centres are present in 2-[ $\alpha$ -(2-ethoxyphenoxy)benzyl]morpholine (Fig. 1A). However, as *I* is prepared by stereospecific synthesis [4], only two enantiomers are present: the *R,R*-(-) enantiomer (*II*) (laboratory code FCE 22071) and the *S,S*-(+)-enantiomer (*III*) (laboratory code FCE 21684). In the tests considered as predictive of antidepressant activi-

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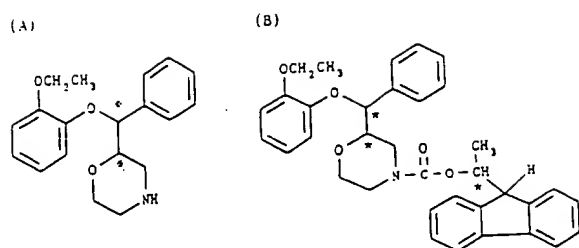


Fig. 1. Structures of (A) reboxetine and (B) its resulting diastereoisomers after derivatization with FLEC.

ty, **III** was found to be more potent than **II** [5]. In the development of a racemic drug it is required that the pharmacokinetic behaviour of each enantiomer be adequately studied [6], as any attempt to establish a plasma concentration–response relationship should be made using only the values of the more active enantiomer or possibly those of both enantiomers, but analysed separately, in order to establish the contribution of each to the therapeutic response and/or to the side-effects. This involves the availability of an analytical method able to determine the two enantiomers in biological fluids [7]. The HPLC method with UV detection available for the determination of **I** in human plasma [8] is characterized by a good limit of quantification (10 ng/ml) but it does not permit the separation of the two enantiomeric forms of the drug.

Different methodologies can be used for enantiomer separation: the precolumn derivatization of the two enantiomers with a chiral reagent to obtain a pair of diastereoisomers, which can then be separated by a conventional achiral column and suitably determined, has wide applications [9]. The applicability of (*R*)-1-(1-naphthyl)ethyl isocyanate [10], (*S*)-1-(1-naphthyl)ethyl isothiocyanate [11,12] and *N*-trifluoroacetyl-L-prolyl chloride [13] as chiral reagents for the derivatization of **I** enantiomers has been investigated. All these compounds reacted with **I** under suitable conditions to give a pair of diastereoisomers. However, although several different columns and several combinations of different mobile phases were tested, no satisfactory separation of the diastereoisomers formed with these reagents was achieved. A different approach for the chromatographic separation of **I** enantiomers involving the use of chiral stationary

phases has been investigated. Several chiral columns have been evaluated so far without success [14].

In this study, optimum conditions were developed for the determination of enantiomers of **I** by reversed-phase HPLC with fluorimetric detection after a derivatization reaction with (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) which occurs in less than 5 min (Fig. 1B). FLEC shows several advantages over other chiral reagents: it forms stable diastereoisomers that can be assayed by automated procedures with high detection sensitivity and generally with good resolution [15].

The HPLC method developed was fully validated down to concentrations of 1 ng/ml in plasma for each enantiomer and used in a preliminary determination of the two enantiomers in plasma samples obtained from a healthy male volunteer who had received a single oral dose of 4 mg of **I** in tablet form.

## EXPERIMENTAL

### Chemicals and solutions

Compounds **I**, **II** and **III**, and the racemic mixture of the two other *RS*- and *SR*-enantiomers (all as methanesulphonate salts) were supplied by the Chemical Development Department of Farmitalia Carlo Erba. All other chemicals and solvents were of analytical-reagent grade from Farmitalia Carlo Erba. Tris buffer, 0.05 *M* (pH 9.1), was prepared by mixing 25 ml of 0.2 *M* Tris solution and 5 ml of 0.1 *M* HCl and diluting to 100 ml with water. Dibasic ammonium phosphate buffer, 0.1 *M* (pH 7.5), was prepared by dissolving 13.2 g of (NH<sub>4</sub>)<sub>2</sub> HPO<sub>4</sub> in about 900 ml of distilled water and adding 85% H<sub>3</sub>PO<sub>4</sub> to pH 7.5 and then water to 1000 ml. Borate buffer, 1 *M* (pH 8.0), was prepared by dissolving 61.8 g of H<sub>3</sub>BO<sub>3</sub> in about 900 ml of distilled water and adding 20% NaOH to pH 8.0 then water to 1000 ml. FLEC reagent was prepared by diluting 1 ml of 18 mM FLEC in acetone solution to 50 ml with acetonitrile. A stock standard solution of **I** was prepared by dissolving a weighed amount of the compound in distilled water. From this solution, which is stable for at least 3 months if stored at 4°C, working standard solutions were

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prepared weekly by dilution with 5 mM H<sub>3</sub>PO<sub>4</sub>. Stock and working standard solutions were stored at 4°C until use.

#### Instrumentation

The HPLC system used in this study consisted of a pump (Model P1000), an autosampler (Model AS 3000) with a 200- $\mu$ l loop, a fluorescence detector (Model FP 820) and a data acquisition system (Winner 386) with LABNET software. Real-time chromatograms were obtained on an integrator (Datajet). All these instruments were supplied by Spectra-Physics (Santa Clara, CA, USA), except the detector, which was purchased from Jasco (Hachioji, Japan). The excitation and emission wavelengths were set at 260 and 315 nm, respectively. The detector was wired to send a 1-V signal to the data system.

Direct probe mass spectra were obtained on a Finnigan Mat (San Jose, CA, USA) Model 4600 instrument operating in the chemical ionization mode, with ammonia as the reagent gas.

#### Chromatographic conditions

The chromatographic separation was carried out on a 250  $\times$  4.6 mm I.D. LiChroCART Supersphere 60 RP-8 (end-capped) reversed-phase column (particle size 4  $\mu$ m) (Merck, Darmstadt, Germany) with a Survival precolumn packed with pellicular ODS (particle size 30–38  $\mu$ m) (Whatman, Clifton, NJ, USA). The mobile phase was 0.1 M dibasic ammonium phosphate buffer (pH 7.5)–tetrahydrofuran (53.5:46.5, v/v). The mobile phase was prepared daily and degassed under vacuum. The separation of the analytes was performed at a flow-rate of 0.5 ml/min. A typical back-pressure was 220 bar.

#### Sample preparation

Blood samples were drawn from a healthy male volunteer participating in a clinical trial of reboxetine, through an intravenous cannula in the forearm and collected in heparinized plastic tubes before and 1, 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72 h after administration. The tubes were immediately centrifuged (1200 g for 10 min) and the plasma stored at –20°C until analysed.

A 1.0-ml aliquot of the plasma sample was placed in a glass-stoppered test-tube and 1 ml of Tris buffer (0.05 M, pH 9.1) was added. The buffered plasma was extracted with 7 ml of diethyl ether by vortex mixing for 1 min then centrifuged at 1200 g for 5 min. The organic phase was carefully separated and transferred into another glass tube where the compounds were back-extracted from the diethyl ether with 0.2 ml of 0.01 M H<sub>3</sub>PO<sub>4</sub> by vortex mixing for 1 min. After centrifugation (5 min at 1200 g), the upper organic phase was separated and discarded. The aqueous phase was mixed with 0.2 ml of 1 M borate buffer (pH 8) and 0.2 ml of the FLEC reagent (0.36 mM in acetonitrile), shaken and left to react for 5 min at room temperature. Then 0.1 ml of a 100 mM aqueous solution of L-proline was added and left to react for 2 min with excess reagent (L-proline gives a water-soluble derivative). The diastereoisomers formed were extracted from the reaction mixture with 3 ml of *n*-hexane (vortex mixer, 1 min) and the organic phase was carefully separated from the aqueous phase. After extraction with 1 ml of acetonitrile, the upper phase (*n*-hexane) was discarded. After further washing with 1 ml of *n*-hexane, the acetonitrile solution was evaporated to dryness on a water-bath (40°C) under a stream of nitrogen. Finally, the residue was dissolved in 0.5 ml of the mobile phase and 200  $\mu$ l of the resulting solution were analysed by HPLC.

#### Determination of quality control and calibration samples

Blank human plasma samples spiked with known amounts of I were analysed by the above procedure. The linearity was evaluated from six calibration graphs with six calibration points prepared and run on six different days in the concentration range 1–270 ng/ml for both enantiomers. The precision and accuracy were evaluated by repeated analyses of the two enantiomers at five concentrations (3.2, 16.0, 40.0, 80.0 and 160.0 ng/ml) in three replicate samples analysed on four different days. All chromatograms obtained were evaluated by peak-area measurement.

### Chromatographic performance

The suitability of the chromatographic system for the analysis of the two diastereoisomers was checked during the validation assay by calculating the column efficiency, the peak symmetry and the resolution factor of the peaks of the two analytes. This evaluation was carried out according to the USP [16] using the System Suitability Test software supplied by Spectra-Physics. The column efficiency was expressed as the number of theoretical plates ( $N$ ). This value must be higher than 10 000 for both enantiomers. The tailing factor ( $T$ ) must be less than 1.35 for **III** and within the range 0.9–1.1 for **II**. The resolution factor ( $R_s$ ) between the diastereoisomer peaks must be  $\geq 1$ .

### RESULTS AND DISCUSSION

The chromatogram obtained from a standard solution of the analytes shows that enantiomers of **I** are sufficiently resolved. The resolution factor between the diastereoisomer peaks was 1.15 and the peak symmetry factors were 1.01 for **II** and 1.12 for **III**. These parameters are characteristic of the day-to-day performance of the separation and can be used to evaluate column ageing or condition and proper mobile phase preparation. If the percentage of tetrahydrofuran is reduced even slightly, the resolution factor improves but the retention times increase drastically. However, the day-to-day reproducibility of the analytical conditions is quite good. The two diastereoisomers of reboxetine elute at about 57 and 59 min. The assignment of the peaks was unambiguous for the two derivatives, since the pure (–)- and (+)-isomers were available. The derivative of **II** eluted first, followed by the derivative of **III**. The enantiomeric purity of FLEC and its chiral integrity under derivatization reaction conditions were determined by derivatizing the reagent with optically pure **II** or **III**. The presence of only one detectable peak under the chromatographic conditions revealed that the racemization of FLEC, if any, was negligible and the reagent used was of very high optical purity.

In order to establish the specificity of the assay

for the *RR*- and *SS*-enantiomers, the separation of the two other enantiomers, *RS* and *SR*, from each other and from *RR* and *SS* was investigated. Under the assay conditions, *RS* and *SR* (the second enantiomeric pair) were separated from each other after derivatization with chiral FLEC (resolution factor = 1.1). The first-eluted peak of the *RS* and *SR* mixture was not separated from the *SS*-enantiomer. However, considering the structure of **I**, the possibility of *in vivo* inversion of configuration at the benzylic position is highly unlikely to occur. Indeed, the formation of the corresponding carbocation would give rise to rearrangement with loss of *O*-ethoxyphenol, and formation of the corresponding carbanion would not cause racemization owing to the absence of the possibility of resonance of the negative charge to form an intermediate species, in which the absolute configuration of the carbanion would be lost, e.g., the formation of a double bond involving the chiral centre, as is the case for profen derivatives. In addition, administration of the *RR*- or *SS*-enantiomers to humans [17] has been shown to result in the absence of detectable peaks at the retention times of the other isomers.

A representative chromatogram for blank human plasma sample is presented in Fig. 2A. The chromatogram exhibits a clean chromatographic window in the region of the two diastereoisomer peaks, although several additional peaks co-eluted until about 90 min after the injection, which prevented us from using an internal standard during the analysis. A significant advantage of using FLEC as derivatizing reagent lies in the remarkable stability of the derivatives formed. The diastereoisomers **II** and **III** can be kept at room temperature dissolved in the mobile phase without apparent degradation. This allowed us to use an autosampler for automatic injection of the samples with an evident increase in the productivity of the system. Considering the HPLC analysis time (90 min) and the total analysis time (including sample preparation), about fifteen samples can be processed in 24 h.

The linearity of this HPLC assay was evaluated from six separate calibration graphs with six calibration points carried out on differ-

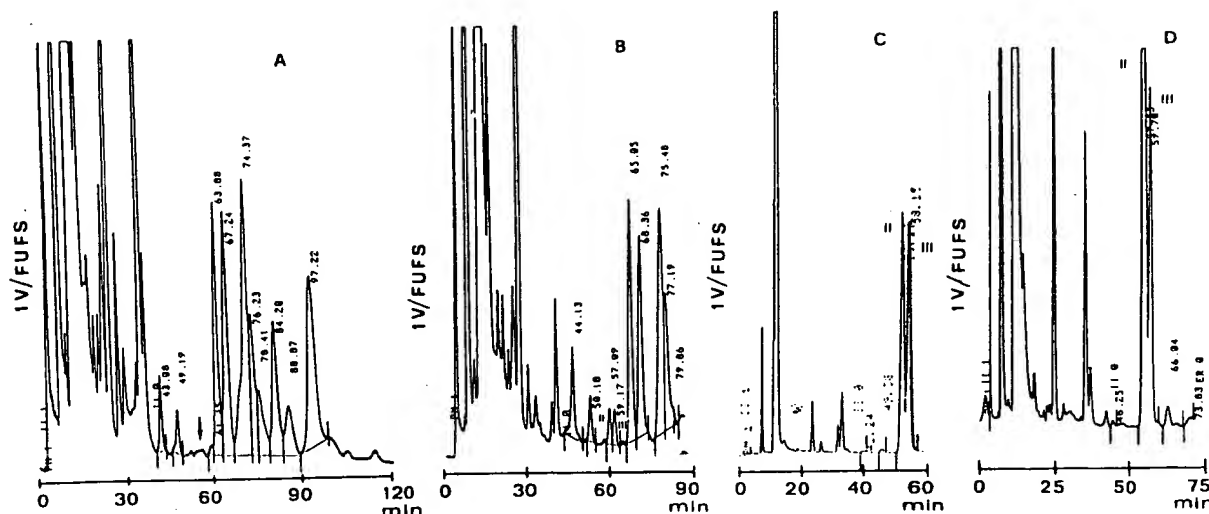


Fig. 2. Chromatograms of (A) blank human plasma (the arrow shows where the two reboxetine diastereoisomers are eluted), (B) human plasma spiked with the lowest concentration of reboxetine (2 ng/ml) used for the calibration graph, (C) human plasma spiked with the highest concentration of reboxetine (540 ng/ml) used for the calibration graph and (D) plasma from a subject dosed with 4 mg of reboxetine (3 h after administration). Peaks: II = (-)-enantiomer; III = (+)-enantiomer.

ent days in the range 2–540 ng/ml of I (1–270 ng/ml for each enantiomer). Calibration graphs were obtained by plotting the measured peak areas (counts) versus the enantiomer concentrations in plasma (ng/ml). Weighted linear regression (weighting factor  $1/x^2$ ) was used to determine the back-calculated standard values or the enantiomer concentration in unknown samples.

Correlation coefficients ( $r$ ) for the regression ranged from 0.9843 to 0.9925 for II and from 0.9785 to 0.9952 for III. The mean calibration graphs obtained were described by the equation  $y = 1222 \cdot 10^3 x - 163 \cdot 10^3$  (slope R.S.D. = 10.3%,  $n = 6$ ) for II and  $y = 1230 \cdot 10^3 x - 71 \cdot 10^3$  (slope R.S.D. = 10.8%,  $n = 6$ ) for III, where  $y$  = peak area (counts) and  $x$  = amount of enantiomer (ng) added to 1 ml of plasma. The back-calculated standard values exhibited an R.S.D. <10.3% for II and <11.6% for III.

The precision of replicate determinations of enantiomers of I evaluated at five concentrations on four different days is presented in Tables I and II. The inter-day precision (expressed as R.S.D.) ranged from 5.1 to 15.1% for II and from 6.7 to 15.0% for III for concentrations ranging from 3.2 to 160.0 ng/ml. At the same

concentrations, the intra-day precision was better than 16.9 and 16.8% for II and III, respectively.

The accuracy of replicate determinations of enantiomers of I evaluated at five concentrations on four different days is summarized in Tables I and II. Accuracy, expressed as the ratio of found to added amount of each enantiomer, ranged from 81.2 to 117.5% for II and from 81.2 to 113.3% for III. The pooled accuracy (inter-day) over the 4-day validation period ranged from 93.7 to 105.0% for II and from 91.0 to 106.7% for III.

The limit of quantification (LOQ), defined as the lowest concentration of an analyte that can be measured with a given level of confidence [18], and chosen as the lowest point on the calibration graph having a back-calculated concentration within 20% of the nominal value, for both enantiomers was 1 ng/ml plasma. For this level the inter-day precision (expressed as R.S.D.) of replicate determinations ( $n = 6$ ) was less than 4% and the mean  $\pm$  S.D. inter-day accuracy (expressed as the ratio of found to added amount) was  $101.5 \pm 3.7\%$  for II and  $98.5 \pm 3.7\%$  for III with a signal-to-noise ratio higher than 10 for both enantiomers (Fig. 2B).

TABLE I  
ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF THE (-)-ENANTIOMER IN HUMAN PLASMA

| Control sample (ng/ml) | Day | n | Accuracy           |                               |                                 | Precision    |                        |                               |
|------------------------|-----|---|--------------------|-------------------------------|---------------------------------|--------------|------------------------|-------------------------------|
|                        |     |   | Mean found (ng/ml) | Mean recovery (%) (intra-day) | Pooled recovery (%) (inter-day) | S.D. (ng/ml) | R.S.D. (%) (intra-day) | Pooled R.S.D. (%) (inter-day) |
| 3.2                    | 1   | 3 | 3.4                | 106.2                         | 103.1                           | 0.23         | 6.8                    | 15.1                          |
|                        | 2   | 3 | 3.7                | 115.6                         |                                 | 0.35         | 9.4                    |                               |
|                        | 3   | 3 | 2.6                | 81.2                          |                                 | 0.10         | 3.8                    |                               |
|                        | 4   | 3 | 3.6                | 112.5                         |                                 | 0.26         | 7.2                    |                               |
| 16.0                   | 1   | 3 | 16.3               | 101.9                         | 105.0                           | 1.81         | 11.1                   | 14.3                          |
|                        | 2   | 3 | 18.8               | 117.5                         |                                 | 3.12         | 16.6                   |                               |
|                        | 3   | 3 | 16.9               | 105.6                         |                                 | 1.65         | 9.8                    |                               |
|                        | 4   | 3 | 15.4               | 96.2                          |                                 | 2.61         | 16.9                   |                               |
| 40.0                   | 1   | 3 | 37.7               | 94.2                          | 93.7                            | 1.17         | 3.1                    | 5.1                           |
|                        | 2   | 3 | 37.9               | 94.7                          |                                 | 3.35         | 8.8                    |                               |
|                        | 3   | 3 | 38.3               | 95.7                          |                                 | 1.94         | 5.1                    |                               |
|                        | 4   | 3 | 36.3               | 90.7                          |                                 | 0.25         | 0.7                    |                               |
| 80.0                   | 1   | 3 | 79.3               | 99.1                          | 98.0                            | 8.76         | 11.0                   | 9.6                           |
|                        | 2   | 3 | 81.7               | 102.1                         |                                 | 2.70         | 3.3                    |                               |
|                        | 3   | 3 | 82.2               | 102.7                         |                                 | 8.75         | 10.6                   |                               |
|                        | 4   | 3 | 70.3               | 87.9                          |                                 | 4.01         | 5.7                    |                               |
| 160.0                  | 1   | 3 | 154.0              | 96.2                          | 100.6                           | 15.27        | 9.9                    | 7.6                           |
|                        | 2   | 3 | 160.5              | 100.3                         |                                 | 15.85        | 9.9                    |                               |
|                        | 3   | 3 | 169.3              | 105.8                         |                                 | 10.34        | 6.1                    |                               |
|                        | 4   | 3 | 160.4              | 100.2                         |                                 | 7.23         | 4.5                    |                               |

Validation data presented in Tables I and II are for the 1:1 mixture only. Quality control data at the more unfavourable ratios of the two enantiomers, a common case in the post-dose plasma samples, were also evaluated. The precision (expressed as R.S.D.) of replicate determinations ( $n = 3$ ) for reconstituted control mixtures of the derivatives II and III, at enantiomeric ratios (II:III) of 50:50, 66.7:33.3, 84.2:15.8 and 96.2:3.8, was  $<0.32\%$  for II and  $<5.26\%$  for III. The accuracy evaluated on the same samples and expressed as percentage recovery ranged from 99.2 to 99.9% for II and from 100.8 to 105.1% for III.

The ammonia ionization mass spectrum confirmed the structure of the reboxetine FLEC derivative. The pseudomolecular ion  $[M + H]^+$

appeared as expected at  $m/z$  550, and it was accompanied by an  $[M + NH_4]^+$  ion at  $m/z$  567.

This method was applied to the determination of the two enantiomers of I in a healthy male volunteer (39 years, 79 kg, 175 cm) who had received a single oral dose of 4 mg of I in tablet form. A typical chromatogram obtained from this subject is shown in Fig. 2D. The peak plasma level ( $C_{max}$ ), the corresponding peak time ( $t_{max}$ ) and the area under the plasma concentration-time curve up to 72 h ( $AUC_{0-72h}$ ) were evaluated for both I enantiomers.  $C_{max}$  and  $t_{max}$  were read directly from the raw plasma data and AUC values were calculated by the linear trapezoidal rule. Peak plasma concentrations of 108 ng/ml (II) and 52 ng/ml (III) were found 1 h after dosing. The experimental areas

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR THE DETERMINATION OF THE (+)-ENANTIOMER IN HUMAN PLASMA

| Control sample (ng/ml) | Day   | n | Accuracy           |                               |                                 | Precision    |                        |                               |
|------------------------|-------|---|--------------------|-------------------------------|---------------------------------|--------------|------------------------|-------------------------------|
|                        |       |   | Mean found (ng/ml) | Mean recovery (%) (intra-day) | Pooled recovery (%) (inter-day) | S.D. (ng/ml) | R.S.D. (%) (intra-day) | Pooled R.S.D. (%) (inter-day) |
| 15.1                   | 3.2   | 1 | 3.2                | 100.0                         | 100.0                           | 0.23         | 7.2                    | 15.0                          |
|                        |       | 2 | 3.5                | 109.4                         |                                 | 0.50         | 14.3                   |                               |
|                        |       | 3 | 2.6                | 81.2                          |                                 | 0.10         | 3.8                    |                               |
|                        |       | 4 | 3.6                | 112.5                         |                                 | 0.35         | 9.7                    |                               |
| 14.3                   | 16.0  | 1 | 15.9               | 99.4                          | 103.7                           | 1.66         | 10.4                   | 12.6                          |
|                        |       | 2 | 18.1               | 113.1                         |                                 | 2.18         | 12.0                   |                               |
|                        |       | 3 | 16.1               | 100.6                         |                                 | 2.08         | 12.9                   |                               |
|                        |       | 4 | 16.5               | 103.1                         |                                 | 2.78         | 16.8                   |                               |
| 5.1                    | 40.0  | 1 | 36.3               | 90.7                          | 91.0                            | 0.92         | 2.5                    | 6.7                           |
|                        |       | 2 | 34.6               | 96.5                          |                                 | 4.12         | 11.9                   |                               |
|                        |       | 3 | 38.0               | 95.0                          |                                 | 2.29         | 6.0                    |                               |
|                        |       | 4 | 36.9               | 92.2                          |                                 | 0.68         | 1.8                    |                               |
| 9.6                    | 80.0  | 1 | 76.0               | 95.0                          | 94.6                            | 8.82         | 11.6                   | 8.1                           |
|                        |       | 2 | 75.6               | 94.5                          |                                 | 1.79         | 2.4                    |                               |
|                        |       | 3 | 79.5               | 99.4                          |                                 | 8.09         | 10.2                   |                               |
|                        |       | 4 | 71.6               | 89.5                          |                                 | 3.75         | 5.2                    |                               |
| 7.6                    | 160.0 | 1 | 162.4              | 101.5                         | 106.7                           | 15.82        | 9.7                    | 7.2                           |
|                        |       | 2 | 165.2              | 103.2                         |                                 | 13.21        | 8.0                    |                               |
|                        |       | 3 | 181.3              | 113.3                         |                                 | 5.99         | 3.3                    |                               |
|                        |       | 4 | 174.1              | 108.8                         |                                 | 6.42         | 3.7                    |                               |

550, and it was  $[^+]$  ion at  $m/z$  567. The determination in a healthy male (175 cm) who had 4 mg of **I** in tablet form obtained from 2D. The peak corresponding peak for the plasma concentration at 72 h ( $AUC_{0-72h}$ ) enantiomers.  $C_{max}$  and the raw plasma data obtained by the linear concentrations of **I** (**III**) were found experimental areas

( $AUC_{0-72h}$ ) were 2123 ng h/ml (**II**) and 847 ng h/ml (**III**).

#### CONCLUSIONS

The method described is sensitive and selective for the determination of enantiomers of **I** in human plasma. It proved to be precise and capable of accurately determining the two enantiomers in the 1-270 ng/ml concentration range. This method is the first enantiospecific analytical methodology developed for the determination of enantiomers of **I** in biological fluids and can be very useful in further clinical pharmacological studies attempting to correlate the individual enantiomer plasma concentrations with the clinical response or the side-effects

observed. Trials to establish such correlations with viloxazine were not successful [19], which is not surprising as no pharmacokinetic studies of the individual enantiomers of viloxazine were carried out, despite the fact that the *S*-isomer is apparently solely responsible for the drug activity, whereas the *R*-isomer is essentially devoid of activity [20].

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